

Antibodies Reactive with Native Lysozyme Elicited by a Completely Synthetic Antigen

(lysozyme residues 64-82/loop)

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ABSTRACT A synthetic peptide consisting of the aminoacid sequence of residues 64-82 of lysozyme, with alanine replacing cysteine as residue 76, was prepared by the solid-phase technique. Mild reduction followed by reoxidation in air of the deprotected peptide led to the formation of a closed loop containing an intrachain disulfide bond. A conjugate consisting of this "loop" attached to multi-poly(DL-alanyl)-poly(L-lysine) elicited, in rabbits and goats, the formation of antibodies capable of reacting with lysozyme and with the loop peptide prepared from it. These immunological interactions can be inhibited by either lysozyme or the loop peptide, but not by the performic acid-oxidized open-chain peptide. Thus, the antibodies elicited by the completely synthetic antigen show specificity toward the "loop" structure (residues 64-80) of native lysozyme.

Studies on molecular aspects of antigenicity have proceeded along two main pathways. In the first approach protein fragments have been screened for immunogenicity or antigenic specificity. In this manner it has been possible to identify relatively small units of several proteins that possess intact antigenic determinants (e.g., 1-5). A second approach has utilized synthetic polypeptide antigens as models for broadening our understanding of various molecular parameters (such as composition, size, shape, accessibility, electrical charge, optical configuration, and steric conformation) that control antigenicity (6, 7).

In the present investigation we have applied a combination of these two approaches. The study is based on previous findings (8, 9) that an isolated fragment of lysozyme [residues 60-83 in the aminoacid sequence of the molecule (10), denoted "loop"], encompassing an independent antigenic determinant (11), is capable, after coupling to a synthetic polymer, of eliciting antibodies that react specifically with the native lysozyme molecule. In this manner we have "grafted" a conformation-dependent antigenic region from a native protein onto a synthetic carrier. The "loop" prepared from peptic digests of native lysozyme contained three half-cystine residues, a situation which can lead to the formation of several isomeric forms as the result of incorrect pairing (9). In the synthetic immunogen described below, the half-cystine residue at position 76 has, therefore, been replaced with alanine. The synthetic loop was prepared by the solid-phase

technique (12) and subsequently conjugated to synthetic multichain poly(DL-alanine)-poly(L-lysine). This material consists of poly(DL-alanine) chains attached to the ϵ -amino groups of a poly(L-lysine) backbone. The above conjugate, although completely synthetic, elicited the production of antibodies which manifest lysozyme specificity and are able to recognize a conformation-dependent determinant in the native protein.

MATERIALS AND METHODS

N α -*t*-Butyloxycarbonyl (Boc)-L-aminoacid derivatives were either purchased from Miles-Yeda, Ltd., or were a gift from Mr. I. Jacobson, Department of Biophysics. The aminoacid side-chains blocked included the β -benzyl (Bz) ester of aspartic acid, the benzyl ethers of serine and threonine, the benzyl thioether (S-Bz) of cysteine, and the nitroguanido (NO₂) group of arginine. ¹⁴C-labeled Boc glycine was prepared from [1-¹⁴C]glycine (New England Nuclear) according to the method of Schnabel (13). All solvents and chemicals used were of analytical grade or the best grade available.

Coupling procedure

Two preparations of the "loop" peptide were synthesized according to the general scheme shown in Fig. 1. The first preparation (A) consisted of the 17 aminoacid residues (Cys⁶⁴...-Cys⁸⁰) comprising the "loop"; the second preparation (B) included two additional residues (Cys⁶⁴...-Ala⁸²). In both preparations Cys-76, present normally in lysozyme, was replaced by Ala, and Gly-67 was ¹⁴C-labeled. Synthesis was begun either at cysteine 80 or alanine 82. For preparation A the Boc derivative of S-Bz cysteine (2.5 mmol) was esterified to 3 g of chloromethylated resin (1.2 mmol Cl per g), and for preparation B the Boc derivative of alanine (2 mmol) was esterified to 4 g of chloromethylated resin (1.7 mmol Cl per g), in the presence of triethylamine (2.5 mmol and 1.8 mmol, respectively), by refluxing in ethanol for 40 hr. The resulting resins contained 0.31 mmol of S-Bz cysteine per g and 0.38 mmol of alanine per g, respectively. In the repeated coupling cycles the Boc group was removed by treatment for 50 min with 66% trifluoroacetic acid, and after the compound had been washed with CH₂Cl₂ and CHCl₃ the peptide hydrochloride was converted to the free base by treatment for 10 min with 10% triethylamine in CHCl₃. After washing, a 3- to 4-fold excess of the appropriate Boc-aminoacid derivative was added in CH₂Cl₂, and an equivalent amount of dicyclohexyl carbodiimide (Fluka) was added. Coupling was allowed to proceed for 2-3 hr. Arginine was added as the

Symbol: A—L, synthetic material consisting of poly(DL-alanine) chains attached to the ϵ -amino groups of a poly(L-lysine) backbone.

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p-nitrophenyl ester in dimethylformamide with a reaction time of 12 hr. Detailed accounts of reagent preparation and rinsing procedures have been published elsewhere (12, 14). The progress of syntheses was monitored by removal of resin samples after the coupling of several aminoacid residues, and their aminoacid analysis after hydrolysis under reduced pressure in 6 N HCl in dioxane for 22 hr at 110°C. The analyses, at various intermediate stages of the two preparations, are given in Tables 1 and 2. The values for half-cystine, obtained as cysteic acid, in the completed "loop" after removal from the resin, were close to theoretical values. However, for the resin-bound peptides these values, determined as S-Bz-Cys, were variable and low.

Cleavage procedure

The peptides were cleaved from the resin by treatment with anhydrous HF for 2 hr at room temperature, in the presence of anisole and $(\text{CH}_3)_2\text{S}$. This treatment brought about complete removal of all the blocking groups. In a typical procedure, we used 3 g of peptidyl resin, 0.5 ml of anisole, 0.5 ml of $(\text{CH}_3)_2\text{S}$, and 15 ml of HF. After removal of the excess HF under reduced pressure, the peptide-resin mixture was extracted three times with ethyl acetate to remove remaining anisole and $(\text{CH}_3)_2\text{S}$, and the peptide was extracted into glacial acetic acid and lyophilized.

In the synthesis of loop preparation B, half of the resin-conjugate obtained in the reaction, namely an amount containing 2 g of the original resin, yielded 730 mg (0.38 mmol) of the deblocked peptide. Since 0.38 mmol/g of alanine was attached to this resin in the first step of the synthesis, the yield of crude peptide was 50%.

Closure of the loop

The peptide as released from the resin was first reduced by exposure to 0.3 M 2-mercaptoethanol in 0.1 M Tris, pH 8.2, for 1 hr at room temperature. The solution was then adjusted to pH 3 with acetic acid, and subjected to gel filtration on Sephadex G-15 (2.5×100 cm) in 0.1 M acetic acid. The fractions under the main peak were diluted to 100 ml, adjusted to pH 8.0, allowed to reoxidize in air (with stirring) at room temperature for 20 hr, and lyophilized. The formed loop was separated from higher molecular weight aggregates on a Sephadex G-25 (fine) column in 0.1 N acetic acid and lyophilized. Reoxidized monomer constituted about 65% of the

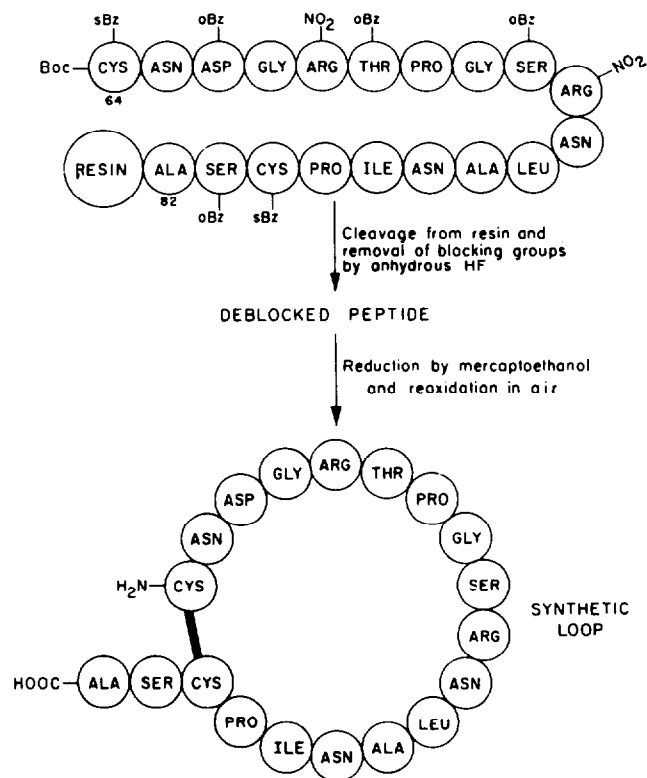


Fig. 1. Scheme of solid-phase synthesis of the loop peptide of lysozyme (preparation B), consisting of residues 64-82. Experimental details are given in the text. Cys-76 was replaced by alanine, Gly-67 was radioactively labeled. For the synthesis of preparation A, consisting of residues 64-80, Cys-80 was attached to the resin.

samples applied to such columns, and the remainder consisted of aggregates of higher molecular weight.

Characterization of the synthetic loop

The aminoacid compositions of the two preparations of the synthetic "loop" are given in Tables 1 and 2. For further characterization of the loop preparations, samples were subjected to hydrolysis by trypsin (1:50 w/w in 0.2 M Tris, pH 8.2, 4 hr at 37°C) and then analyzed by diagonal paper

TABLE 1. Aminoacid composition of the synthetic loop preparation A and its intermediary stages

	After attachment of 5 residues		After attachment of 10 residues		Complete loop		Off-diagonal peptides from tryptic digest			
	Found	Calcd for 76-80	Found	Calcd for 71-80	Found	Calcd for 64-80	Found	Calcd for 64-68	Found	Calcd for 74-80
Arg			0.85	1	1.64	2	1.1	1		
Asp	0.98	1	1.84	2	3.2	4	2.0	2	2.0	2
Thr					0.7	1				
Ser			0.90	1	0.9	1				
Pro	1.04	1	1.20	1	2.2	2			1.5	1
Gly			1.06	1	1.66	2	1.2	1		
Ala	1.09	1	1.02	1	1.0	1			1.0	1
Cys	0.65*	1	0.60*	1	1.02*	2	0.9†	1	1.0†	1
Ile	1.00	1	1.10	1	1.0	1			1.2	1
Leu			1.00	1	1.0	1			1.3	1

* Determined as S-Bz-cysteine (low and variable values were obtained on several samples of resin-bound peptides).

† Determined as cysteic acid.

TABLE 2. Aminoacid composition of the synthetic loop preparation B and its intermediary stages

	After attachment of 5 residues		After attachment of 13 residues		Complete loop		Tryptic peptides					
							Off-diagonal peptides				Basic peptide on the diagonal	
	Found	Calcd for 78-82	Found	Calcd for 70-82	Found	Calcd for 64-82	Found	Calcd for 64-68	Found	Calcd for 74-82	Found	Calcd for 69-73
Arg			0.98	1	1.91	2	1.07	1			0.96	1
Asp			2.00	2	4.06	4	1.82	2	2.10	2		
Thr					0.84	1					0.93	1
Ser	0.78	1	1.63	2	1.86	2			0.91	1	0.90	1
Pro	1.15	1	2.06	2	2.07	2			1.10	1	1.10	1
Gly			1.20	1	2.24	2	1.00	1			1.00	1
Ala	1.00	1	2.04	2	1.97	2			1.90	2		
Cys	0.89*	1	0.85*	1	1.90†	2	1.00‡	1	1.09‡	1		
Ile	1.12	1	1.00	1	1.05	1			1.00	1		
Leu			1.29	1	1.15	1			1.00	1		

* Determined as *S*-Bz-cysteine (low and variable values were obtained on several samples of resin-bound peptides).

† Determined as cysteine.

‡ Determined as cysteic acid.

electrophoresis (15) at pH 3.5. The aminoacid analyses of the off-diagonal peptides obtained after performic acid oxidation are listed in Tables 1 and 2. These data are consistent with the correct sequences around the cysteine residues. The pentapeptide Thr⁶⁹-Arg⁷³ (see Fig. 1) resulting from the cleavage at the two arginine residues was also isolated by paper electrophoresis of the tryptic peptides of preparation B (Table 2).

The molecular weight of the synthetic loop preparation was evaluated in an analytical ultracentrifuge, using a double-sector cell and interference optics (16).

Attachment of the synthetic loop to A---L

The synthetic loop was attached to multi-poly(DL-alanyl)-poly(L-lysine), here designated A---L. Under these conditions synthetic loop preparation A did not become attached, whereas the conjugate prepared with loop preparation B contained 9.9% (w/w) of the loop peptide. This conjugate is designated synthetic Loop-A---L.

Preparation of immunoadsorbent

The support for the immunoadsorbent was Sepharose 4B. An attempt to bind loop preparation A to the activated Sepharose was unsuccessful. However, addition (17) of a short polyalanine chain to the amino terminus of the loop resulted in a derivative that could be bound to the activated Sepharose through the free amino group. Loop preparation B, in which the terminal carboxyl group is not contiguous to the loop, could be bound to Sepharose without previous alanylation. The activated Sepharose was allowed to react with ethylene diamine and the loop was coupled with the aid of 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide (18). In this case the binding occurred through a free carboxyl group of loop B. Preparation of the lysozyme immunoadsorbent was described elsewhere (9).

Immunological procedures

Rabbits and goats were immunized by multisite intradermal injections (5-10 mg) in complete Freund's adjuvant. Antibodies were isolated on the appropriate immunoadsorbents

(9). Antigen-binding capacity was measured (9) with the use of either ¹²⁵I-labeled lysozyme or the synthetic loop containing [¹⁴C]Gly-67. In these experiments the anti-loop antibodies were prepared in goats and, therefore, rabbit anti-goat IgG served for precipitation of the Ag-Ab complexes. Studies on the inactivation of modified bacteriophage preparations employed loop-T₄ (9) or lysozyme-T₄ conjugates prepared according to Haimovich *et al.* (19). Each of the modified bacteriophage preparations (about 1000 plaque-forming units) was incubated for 2 hr with different dilutions of the antiserum, and was subsequently plated with bacteria for monitoring of the surviving phages. Fluorescence measurements and fluorometric titrations were performed (20) with a dansylated loop preparation with the aid of the Turner Spectrofluorometer, model 210.

RESULTS

Characterization of the synthetic loop

The aminoacid compositions of the two preparations of the synthetic loop (Tables 1 and 2) show good agreement between the expected values and the experimental data. Both preparations contained only two cysteinyl residues, so that there was only one possibility for formation of an intrachain disulfide bond. This assumption was verified by tryptic digestion followed by performic acid oxidation. The aminoacid analyses of the isolated peptides were in accord with the expected data for the composition of the relevant peptides (Tables 1 and 2).

To exclude the possibility that the synthetic preparation consisted of a circular molecule containing more than one unit of the synthetic peptide, we subjected the material to ultracentrifugal analysis. Because the open-chain synthetic peptide was available (the monomeric performic acid-oxidized synthetic loop), the molecular weight of the closed loop could be estimated by direct comparison in the ultracentrifuge (16). The advantage of this approach over the customary procedures for molecular weight determinations is that the two materials are compared under identical conditions of angular velocity and temperature. Solutions of the synthetic loop and

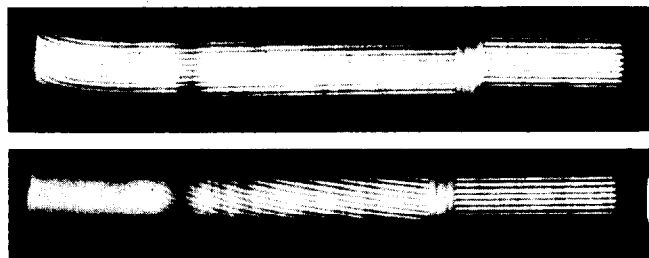


FIG. 2. Ultracentrifugation patterns illustrating a differential method for comparison of molecular weights (16). Above, a solution of synthetic loop in buffer was placed in one sector of the double-sector cell, and the other sector contained a solution of performic acid-oxidized synthetic loop of exactly the same concentration. Below, control experiment, with the synthetic loop solution placed in one sector, whereas the other sector contained the buffer. The photographs were taken after 24 hr at 40,000 rpm at 20°C.

the performic acid-oxidized synthetic loop in 0.15 M sodium chloride–0.02 M sodium phosphate, pH 7.2, of exactly equal concentrations on the basis of radioactivity measurements, were prepared and subjected to equilibrium ultracentrifugation at 40,000 rpm in a double-sector cell. After 24 hr, interference optics revealed straight horizontal fringes (Fig. 2A), which indicates that the molecular weights of the two materials were very similar. In a control experiment, comparison of the closed synthetic loop with solvent in the double-sector cell gave, as expected, typically curved fringes (Fig. 2B). Thus, it may be concluded that the synthetic loop is indeed a monomer of the peptide with an intrachain disulfide bond.

Antibodies to the synthetic loop

Loop-A–L (from preparation B) was used for immunization of rabbits and goats. The concentration of antibodies capable of interacting with lysozyme in the antisera from both species was approximately 0.1 mg/ml. These antibodies could be purified on immunoadsorbents containing either lysozyme or synthetic loop. In most of the experiments described below, antibodies purified on the lysozyme immunoadsorbent were used. The injection of Sepharose-bound synthetic loop (preparation A) also led to the production of antibodies with lysozyme specificity, but at very low levels.

Characterization of the antibodies

The antibodies against the synthetic loop were compared, by several immunological techniques, with anti-lysozyme antibodies and/or antibodies obtained by immunization with a conjugate of A–L with the natural loop peptide prepared from peptic digest of lysozyme (8, 9).

Antigen-Binding Capacity. The antibodies were compared with respect to their capacities to bind either radioactively labeled lysozyme or the radioactive synthetic loop. The binding of lysozyme is most efficient with its homologous antibodies (Fig. 3A). Antibodies against both natural loop and synthetic loop can bind the protein, but less efficiently. On the other hand, binding of the synthetic loop (Fig. 3B) is most efficient with antibodies elicited by the synthetic loop conjugate, whereas the two other antibody preparations show lower antigen-binding capacities.

Inactivation of Modified Bacteriophage Preparations. Fig. 4 shows the inactivation of either lysozyme-bacteriophage or

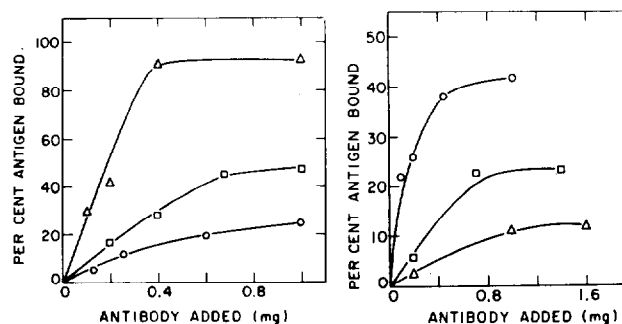


FIG. 3. Antigen-binding capacity of the different antibody species. Binding of ^{125}I -labeled lysozyme (A) and of synthetic loop labeled with $[1-^{14}\text{C}]$ glycyl-67 (B) to: anti-lysozyme antibodies (Δ); antibodies obtained by immunization with the conjugate of the natural loop of lysozyme (\square); and antibodies obtained by immunization with the conjugate of the synthetic loop (\circ).

loop-bacteriophage conjugates by antiserum against the synthetic loop. Although the inactivating efficiency of the antiserum is low, it is clear that the serum is capable of inactivating both bacteriophage preparations, and that the rates of inactivation are similar, as indicated by the slopes of the curves.

Energy Transfer to Dansylated Loop. In a previous publication (20) we have reported that, by interaction of anti-loop antibodies with a loop preparation dansylated at its amino terminus, an excitation energy transfer from the antibodies to the dansyl group is observed, which is manifested in enhanced fluorescence. The same effect is brought about by the antibodies to the synthetic loop, as shown in Fig. 5. From the data of this fluorometric titration, the binding parameters of these antibodies were calculated (20). The association constants of two preparations of the antisynthetic loop antibodies were 1.1×10^6 and $0.6 \times 10^6 \text{ M}^{-1}$, the homogeneity indices 0.88 and 1.05, respectively. A value of $3.2 \times 10^6 \text{ M}^{-1}$ was previously (20) obtained for the binding constant of antibodies prepared against the conjugate of the natural loop.

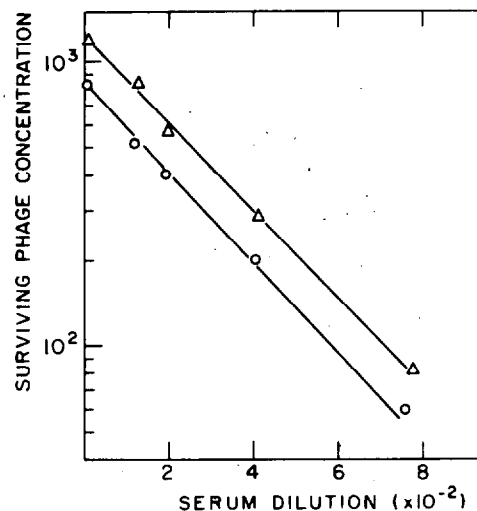


FIG. 4. Inactivation of chemically modified bacteriophage T4 preparations by antiserum to the synthetic loop conjugate. Inactivation of lysozyme-bacteriophage conjugate (Δ) and loop-bacteriophage conjugate (\circ) as a function of antiserum dilution.

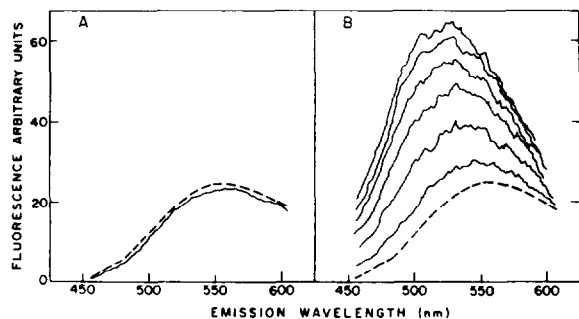


Fig. 5. Effect of antibodies on the emission spectrum of dansyl loop. The broken line represents the spectrum of dansyl loop at a concentration of $7.5 \mu\text{M}$. A, emission spectra of the same dansyl loop solution in the presence of normal goat IgG ($0.50 \mu\text{M}$). B, emission spectra in the presence of increasing amounts of antibodies elicited by the synthetic loop conjugate. The antibody concentrations in the various samples were 0.08, 0.24, 0.40, 0.57, 0.73, and $0.88 \mu\text{M}$, in ascending order. Excitation at 280 nm in 0.01 M phosphate buffer (pH 7.2)–0.15 M NaCl.

The increase in fluorescence induced by the interaction with the antibodies could be specifically inhibited by either lysozyme or the loop peptide, as shown in Fig. 6, but not by the open-chain loop peptide. The synthetic loop has an inhibitory capacity identical to that of the natural loop prepared from a lysozyme digest. In this system, therefore, the antibodies to the synthetic loop behave similarly to the anti-loop antibodies described previously (9).

DISCUSSION

Several cases of cross-reactions between antibodies to synthetic materials with natural antigens have been reported in recent years (see ref. 7). Thus, e.g., antibodies to synthetic uridine-A-L reacted with single-stranded DNA (21), and ordered copolymer (Pro-Gly-Pro)_n, which exists in a triple helical conformation, elicited antibodies reactive with collagens of different species (22). In the last two cases the recognition of the native material by the antibodies involved general features, such as the presence of purine nucleotides in DNA, or the gross geometry of the collagen molecule. The antibodies described in the present study recognize exclusively a unique region of a native protein (hen egg-white lysozyme).

Two preparations of the lysozyme loop were synthesized. Preparation A consisted only of the 17 amino acids corresponding to the sequence of the loop in the native lysozyme molecule. It therefore contained terminal carboxyl and amino groups, both belonging to cysteinyl residues, spatially close to one another. The finding that this preparation did not undergo chemical attachment to the carrier suggested that the presence of a dipolar ion was a restricting factor. Preparation B was, therefore, synthesized, with two additional residues on its carboxyl terminus. In this case there was no difficulty in binding the loop to the carrier through the "free" carboxylic groups of the loop and the amino groups of A-L.

The antibodies prepared by immunization with the conjugate of the synthetic loop exhibit, according to several criteria, properties similar to those of antibodies elicited by an equivalent conjugate of the natural loop (Figs. 3–6). According to the results of the antigen-binding experiments, these antibodies react more efficiently with the loop than with intact lysozyme.

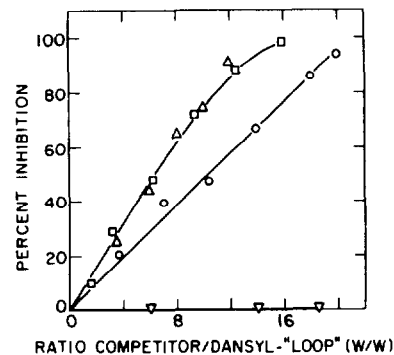


Fig. 6. Inhibition of the enhanced fluorescence of a mixture of dansyl loop ($2.5 \mu\text{M}$) and antisynthetic loop antibodies ($0.4 \mu\text{M}$), by lysozyme (\circ), natural loop (Δ), synthetic loop (\square), or performic acid-oxidized synthetic loop (∇). The extent of inhibition was calculated from the decrease in the fluorescence at 520 nm.

In the transfer reaction of the excitation energy from the antibodies to the dansylated loop, antibodies to the synthetic loop bring about the same enhancing effect as the antibodies to the natural loop. When the data obtained by this technique were quantitatively analyzed, the homogeneity index and the association constant of the antibodies to the synthetic loop were found to be quite similar to the values reported (20) for antibodies to the natural loop. Likewise, the specificity of the reactions with the two types of antibodies is identical (Fig. 6). The anti-loop antibodies, obtained with the conjugate of A-L and either the synthetic or the natural lysozyme loop, did not react with loop derivatives in which the disulfide bridge was opened. The loop specificity thus depends strongly on its conformation.

The loop that is prepared from the lysozyme molecule contains, in addition to the two cysteinyl residues that form the disulfide bridge, an additional cysteine residue at position 76 (9). Since the isolation of this loop included procedures of reduction and reoxidation (9), the possibility of formation of an incorrect disulfide bond in the loop could not *a priori* be disregarded, even though it was shown (9), by the use of the diagonal paper electrophoresis technique, that in 70–80% of the reoxidized natural loop the disulfide bond found was identical to the bond present in intact lysozyme. In the synthetic loop this difficulty was overcome by replacing Cys-76 with an alanine residue. Closure to form a larger loop, which contained two or more peptide units, connected through disulfide bonds, was ruled out by ultracentrifugal analysis, which showed that the molecular weight of the loop is equal to that of the performic acid-oxidized peptide. Thus, the synthetic loop consists of a cyclic monomer and closely resembles the loop that occurs in native lysozyme.

We have shown in this investigation that a synthetic 19-residue peptide, containing a disulfide bond, folds to produce a conformation similar to that characteristic of this sequence in the native protein. Consequently, the antibodies elicited by the synthetic conjugate are specifically directed toward a conformation-dependent determinant.

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